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13. SUPPLEMENTARY NOTES

14. ABSTRACT:
This grant will provide critical data on tolerance and toxicity of cell dosing and numbers of permissible spinal cord injections. Rigorous experiments in Aim 1 of our grant have demonstrated that, even though the porcine spinal cord seems to tolerate escalating numbers and volumes of injections, the severity of acute transient morbidity should not be neglected. Moreover, escalating numbers and volumes of injections seem to be associated with lack of accuracy and reflux. Histological analysis will further assess morbidity by determining whether there is a reduction in the number of motor neurons or not in grafted areas in comparison with intact segments. Histological analysis will also determine whether reflux occurs with volume escalation as well as with fast (hand-held) injections.
At the same time, little is understood about the appropriate immunosuppressive therapy for spinal cord stem cell transplant recipients. In our ongoing human trial, aggressive immunosuppressant therapy has formed the single biggest source for adverse events. Aim 2 will help us to minimize immunosuppression, preventing needless complications.

15. SUBJECT TERMS
ALS, spinal cord, stem cell, swine, therapy, transplantation.

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INTRODUCTION:

"The present application will refine critical details required for successful cell transplantation.
Aim 1 (Optimal Surgical Technique) will provide critical data on tolerance and toxicity of cell dosing and numbers of permissible spinal cord injections. Aim 2 (Graft Rejection) will provide critical data on graft rejection and appropriate immunosuppression for human spinal cord stem cell transplantation."

Work in Year 1 has focused mainly on Aim 1.

The approved Statement of Work for such aim states the following:

- "**Aim 1: *Optimal Surgical Technique*** is required for both accuracy and safety.

The Svendsen Laboratory has extensive expertise in the production and propagation of neural progenitor lines. Ongoing preliminary studies have allowed the Boulis and Svendsen laboratories to develop Standard Operating Procedures (SOPs) for the transfer of healthy cells. Cells will be shipped overnight. An individual from the Svendsen laboratory will fly to Atlanta the day before surgeries. On the next day, cell will be prepared for injections and viability counts will be performed in the Boulis Laboratory, prior to transplantation. Surgeries will be performed in the Division of Animal Resources (DAR) at Emory University. Each pig surgery takes approximately 3.5 hours, making it possible to perform 2-3 surgeries in a day. Animals will be euthanized after 21 or 30 days. At the time of necropsy, animals will be sedated and perfused with heparin, followed by Paraformaldehyde. Fixed tissue will be sectioned. Tissue will be analyzed for histological morbidity. Alternating tissue sections will be shipped to the Svendsen Laboratory for graft identification / migration."

- **Milestones**

Year 1:

"Complete analysis of reflux and transient morbidity with number and volume of injection of hNPCs (Boulis).
Create a cell bank of astrocyte restricted precursor IPS cells (Svendsen)."

BODY:

In Year 1 we have completed all surgical procedures pertaining to Aim 1 (Optimal Surgical Technique). Adjustments to the original aims and milestones were made to accommodate the logistics of animal OR scheduling and cell production, as well as adjustments to the subaims to reflect observations that had occurred in the window between grant submission and project initiation. A total of 45 animals were used.

(Boulis Laboratory)

a. **"Number-Escalation - We hypothesize that there is a *number* of injections that forms a threshold for permanent neurological morbidity. We will perform a dose escalation of 10, 20, and 30 unilateral cervical injections."** In this aim, we performed a dose escalation of number of injections (20, 30, and 40 bilateral cervical injections; n = 5 / group). *(Although the original grant proposed unilateral injections for the purpose of within subject comparisons, (20) bilateral injections had already proven to be tolerable in previous studies conducted by our group after grant submission. We, therefore, chose to proceed with bilateral injections.)*

Although anticipated that multiple injections concentrated in a small space (inter-graft distance of 2 mm) could reach a threshold for parenchymal damage, ***transient morbidity did not exceed post-operative days 3-4.*** Moreover, all animals recovered to baseline behavior by post-operative day 14 (Figure 1).

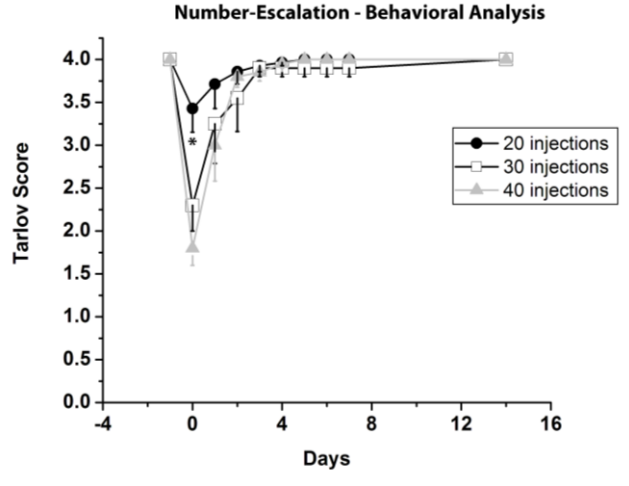

Fig.1: Behavioral analysis of escalating number of injections **assessed by the average Tarlov score at different post-operative days**. Day 0 (*p = 0.002)

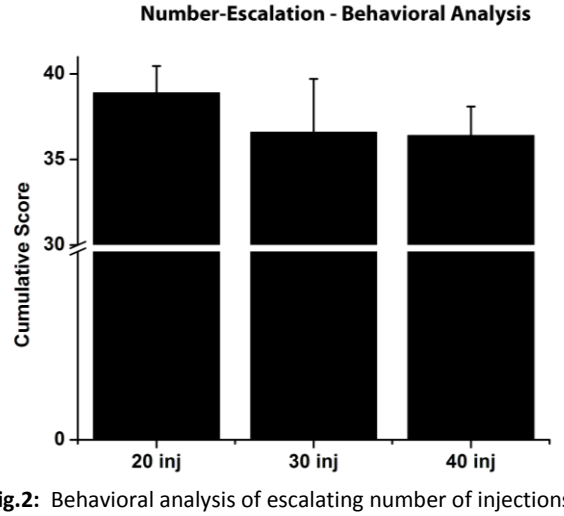

Fig.2: Behavioral analysis of escalating number of injections **assessed by cumulative scores**. (NS; p = 0.114)

Even though it decreased as the number of injections escalated, ***behavioral morbidity assessed with a cumulative score[1] (Figure 2) did not show statistically significant differences*** (NS; p = 0.114).

[1] Cumulative score is the sum of Tarlov scores below baseline for each day of the experiment. The maximum cumulative score for a given animal is 40 (day -1 + day 0 + day 1 + day 2 + day 3 + day 4 + day 5 + day 6 + day 7 + day 14).

The severity of acute transient morbidity, however, should not be neglected, as demonstrated in **Figure 1** (post-operative days 0 to 3-4; Day 0 ; *p = 0.002).

Histological morbidity using stereology to assess the density of motor neurons in sections with grafts is ongoing (Figure 3).

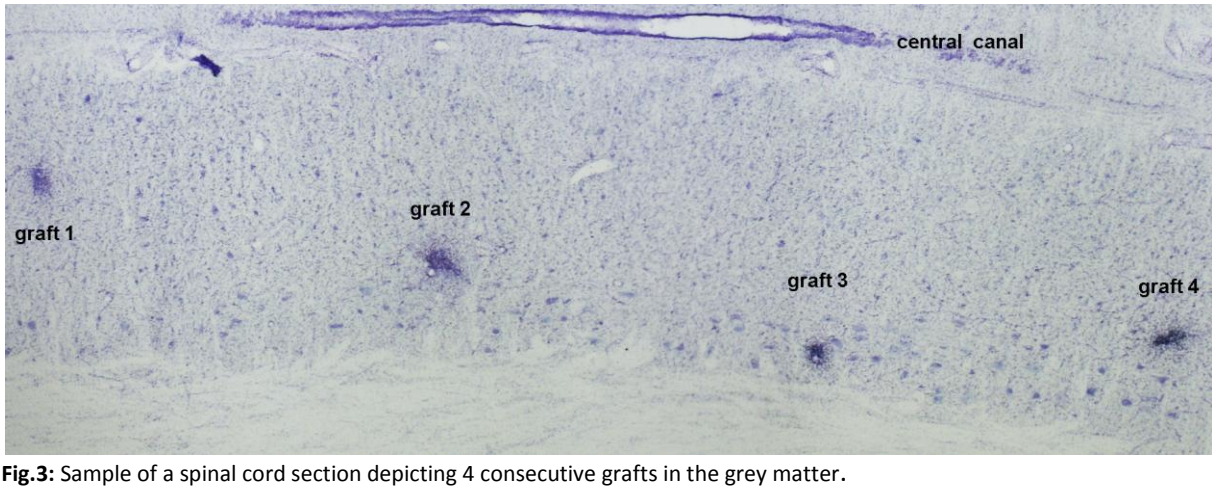

Fig.3: Sample of a spinal cord section depicting 4 consecutive grafts in the grey matter.

b. **"<u>Volume-Escalation</u> - There is a threshold for morbidity in terms of *volume* of injections. Using a subtoxic dose from *Aim 1a*, we will escalate volume (10, 25, and 50 microliters/injection)."** In this aim, we performed a dose escalation of volume of injections (10, 25, 50, and 75 microliters per injections with a total of 20 bilateral cervical injections; n = 5 / group and n = 2 for the 75ul group).

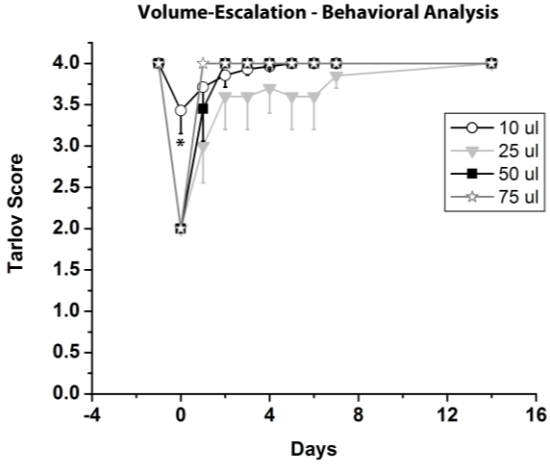

Fig.4: Behavioral analysis of escalating volume of injections **assessed by the average Tarlov score at different post-operative days**. Day 0 (*p = 0.003)

Once again, although anticipated that high volumes of cells could result in compression injuries, which could clinically result in paresis, **transient morbidity did not exceed post-operative days 4-5 in the majority of the groups.** All animals recovered to baseline behavior by post-operative day 14 (Figure 4).

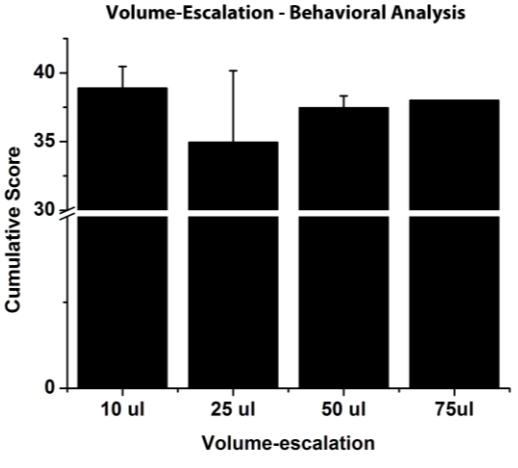

Fig.5: Behavioral analysis of escalating volume of injections **assessed by cumulative scores**. (NS; p = 0.076)

Behavioral morbidity assessed with a <u>cumulative score</u> (Figure 5) did not show statistically significant differences (NS; p = 0.076).

Due to the physical limitations of the parenchyma with respect to increasing volumes (see calculations below), we strongly believe that volumes higher than 25ul may result in reflux. Histological analysis to confirm/assess this hypothesis is ongoing.

The volume of an injected segment of the pig spinal cord (considering 10 unilateral or 20 bilateral injections roughly spaced by 2.5mm) is equal to 1,206.5 mm^3 (7.7mm x 5.7mm x 30mm – shape of an elliptical prism). ***By injecting 20 x 50ul, we are injecting an extra volume of 1,000 mm^3 in that segment.***

20 x 10ul = 200 mm^3 (reasonable volume)

20 x 25ul = 500 mm^3 (reasonable volume, but morbidity?)

20 x 50ul = 1,000 mm^3 (reflux?)

20 x 75ul = 1,500 mm^3 (reflux?)

We have also observed that the cord acutely swells with volume escalation making dural closure virtually impossible (data not shown).

c. **"<u>Floating vs. Rigid Cannula</u> (new <u>Platform vs. Hand-held Injections</u>) - Pulsation of the spinal cord occurs with ventilation as well as with the heartbeat. We hypothesize that this movement with respect to a rigid cannula anchored to the spine results in added trauma during the course of pumped graft injection."** *This aim was adapted to assess the use of* ***<u>"platform vs. hand-held" injections</u>*** *instead. This "adapted" aim also served to address "slow vs. fast" injections with high volumes (to prove the argument that slow injections prevent reflux and morbidity).* In this aim, we performed a dose escalation of volume of injections (10, 25, and 50 microliters per injections with a total of 20 bilateral cervical injections; n = 3 / group). As an additional part of this aim, we also performed an in vitro testing to assess <u>cell viability at increasing infusion rates</u>, before proceeding with in vivo experiments using higher volumes and faster infusion rates.

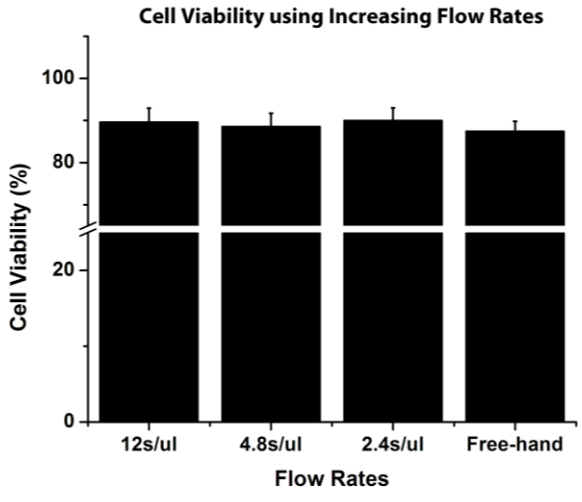

Fig.6: Increasing flow rates and free-hand (i.e., hand-held) loading and delivery of cells did not reduce cell viability (NS; p = 0.542). Baseline viability before these experiments was equal to 94.73%.

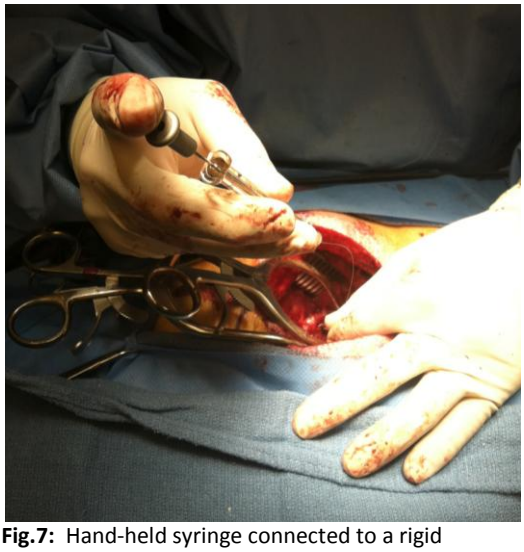

Fig.7: Hand-held syringe connected to a rigid needle.

Before proceeding with the comparison of platform vs. hand-held injections using higher volumes and faster infusion rates, we performed an in vitro testing to assess <u>cell viability at increasing infusion rates</u>. We concluded that increasing flow rates and free-hand (i.e., hand-held) loading and delivery of cells did not reduce cell viability (NS; p = 0.542) in comparison to baseline viability before loading/delivery of cells (94.73%) - (Figure 6).

Although anticipated that performing injections with a rigid cannula (in our case, a hand-held Hamilton syringe connected to a rigid needle of the same gauge as the floating cannula one – Figure 7) using the maximum volume/number of injections could result in clinical morbidity, **transient morbidity did not exceed post-operative day 1 with hand-held injections** (Figure 8).

Behavioral morbidity assessed with a <u>cumulative score</u> (Figure 9) did not show statistically significant differences (NS).

In summary:

- Hand-held injections were performed in an incredibly quicker manner in comparison with injections using the platform.
- Accuracy and reflux were identified by the surgeons as the main potential challenges of such technique.

Based on these two observations, we can conclude that ***slow injections may prevent reflux but not necessarily reduce morbidity.***

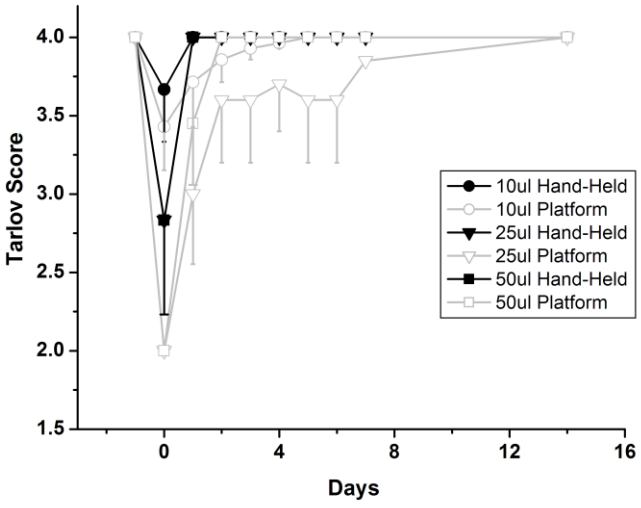

Fig.8: Behavioral analysis of hand-held (black) vs. platform (grey) injections **assessed by the average Tarlov score at different post-operative days**.

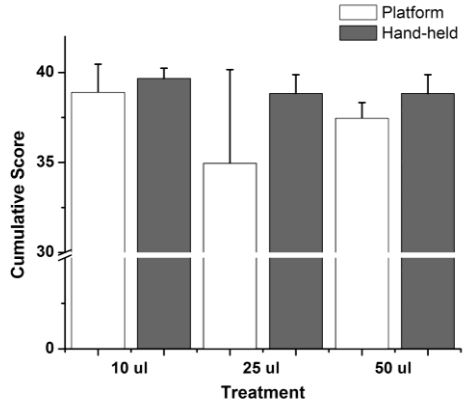

Fig.9: Behavioral analysis of hand-held (grey) vs. platform (white) injections **assessed by cumulative scores**. (NS)

d. **"<u>Uni vs. Bilateral Injections</u> - *Unilateral* injection is less morbid than bilateral injection. We hypothesize that bilateral injuries of the spinal cord are far more likely to result in functional deficits than unilateral injuries."** Our initial experience in the human spinal cord has shown that multiple and bilateral lumbar injections can be accomplished safely. In addition, recent studies conducted by our group after grant submission have concluded that cervical bilateral injections did not increase morbidity in minipigs (*not yet published*). As originally proposed, we expected that bilateral injections would generate higher morbidity than unilateral injections. However, in the absence of permanent morbidity, bilateral cervical injections should be considered as a means to amplify the preservation of ventilatory drive by treating both phrenic motor neuron pools. We, therefore, concluded that it was unnecessary to pursue this aim.

e. **"<u>Staggered vs. Symmetric Injections</u> – We hypothesize that staggered injections (which avoid penetrating the same coronal plane bilaterally) will induce less morbidity than symmetric injections and therefore carry less morbidity than *symmetric* injection."** As we started escalating the number of injections beyond 20 (in subaim 1a), staggering injections and reducing the interval space of injections became a necessity. Moreover, the higher the number of injections the more likely is that the vasculature will interfere with a symmetric / linear pattern of injections. We, therefore, concluded that it was unnecessary to pursue this aim.

(Svendsen Laboratory)

<u>Create a cell bank of astrocyte restricted precursor IPS cells</u>

The Svendsen Laboratory provided cells for all surgical procedures in Aim 1, Year 1. The Svendsen Laboratory has identified that the original cell concentrations proposed in the grant were prohibitive and decided to proceed with what they called "a more clinically relevant concentration" of 10E4cells/microliter. They also stated that by doing so, they would be able to provide cells at similar cell passage numbers for all of the phases of the study.

The Svendsen Laboratory has also focused Year 1 on the production of pig iPS cells, as well as the generation of fetal pig-derived neural progenitors.

A detailed description of their key research accomplishments, as well as reportable outcomes will follow.

PIG IPSC PRODUCTION: Previous human and pig iPSC generation techniques have involved the use of lentiviral or retroviral delivery using a combination of "six pack" of reprogramming vectors Oct4, Lin28, Sox2, Klf4, c-Myc, and Nanog. While integrating viral methods of iPS production are rapid and robust for developing iPS cell lines from starting tissues, they are potentially riskier due to a greater risk of tumor formation from transplantation of iPSC-derived cells. Transgene integration into the DNA of the cell may interfere with subtle processes within the cell that may be important components of any cellular phenotype observed in during transplantation. Non-integrating methods, such as plasmid-based and Sendai single-stranded RNA viruses, avoid random insertion of DNA into the genome overcoming the transgene integration problem.

Figure 1
Episomal plasmid reprogramming

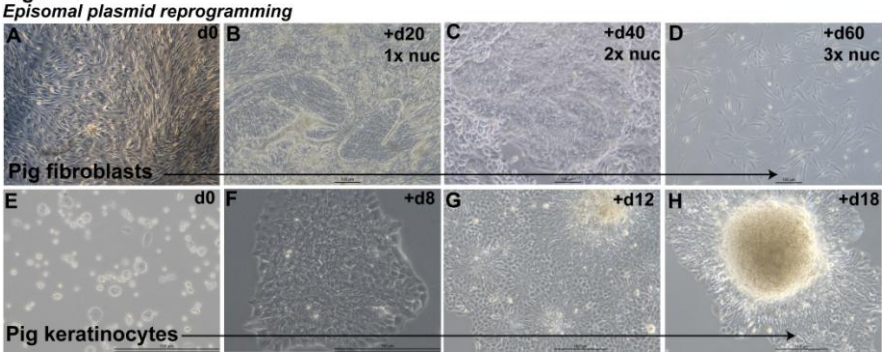

Fig. 1: Attempts at reprogramming Yucatan mini-pig dermal fibroblasts and keratinocytes using episomal plasmid-based approach. Phase contrast images showing morphology of (A-D) fibroblasts and (E-H) keratinocytes along a timeline in days after single (for keratinocytes) or multiple (for fibroblasts) plasmid nucleofection of cells. (A and E) Untransfected cells.

To generate transgene-free Yucatan minipig iPSC lines, we first utilized oriP/EBNA1 (Epstein-Barr nuclear antigen-1)-based reprogramming vectors that utilize episomal expression of the 6 pluripotency factors and avoid integration events[1, 2]. This approach works robustly in our hands for reprogramming human somatic tissues to iPSCs as we have successfully reprogrammed > 25 human iPSC lines. The episomal vectors are gradually lost from proliferating cells in the absence of selection and provide stable extrachromosomal replication only once per cell cycle. They do not suffer from the "footprint" problem experienced when techniques are used to remove virally inserted DNA after reprogramming. We attempted this with adult Yucatan minipig fibroblasts and keratinocytes. Amaxa Dermal Fibroblast Nucleofector Kit was utilized to generate the episomal-piPSC lines. Briefly, sufficient numbers of fibroblasts (1.0 x 10^6cells per sample) were harvested and centrifuged at 200xg for 10 minutes. The cell pellet was resuspended carefully in Nucleofector® Solution combined with using episomal reprogramming factors, OCT3/4, SOX2, KLF4, L-MYC, and LIN28, and shRNA to p53 vectors (Addgene). The cell/DNA suspension was transferred into the Nucleofector® and different programs were applied and tested. The sample was gently transferred on to pre-warmed MEF feeder or Matrigel coated plates with the media. The pig dermal fibroblasts were extremely difficult to reprogram with this technique, even after multiple nucleofections (**Fig. 1B, C and D**). They formed transient intermediates that appeared to be of non-pluripotent cell types, never obtained an ideal pig iPSC morphology (**Fig.1C**), and ultimately senesced (**Fig. 1D**). The pig skin keratinocytes initially appeared to be more amenable to plasmid-based reprogramming, as evidenced by typical iPSC morphology only 8 days after nucleofection (**Fig. 1F**). However, even these cells remained in this state transiently and progressed to form neural rosette- (**Fig. 1G**) and neurosphere-like structures (**Fig. 1H**). **We were unable to "lock" and maintain them in a pluripotent state. Thus, we postulated following possibilities that could result in lack of reprogramming in minipig somatic cells to iPSCs using this approach: (i) a younger source of fetal somatic tissue may be required, and/or (ii) stronger and sustained expression of pluripotency reprogramming factors is required for longer duration in Yucatan minipig cells to turn on the endogenous pluripotency program and achieve complete reprogramming.**

Recent evidence implies that iPSCs retain residual epigenetic memory of their tissue of origin, which may facilitate iPSC differentiation toward parental cell lineages. As a result we isolated and expanded neural progenitor cells (NPCs) from Yucatan minipig fetal cortex and spinal cord for reprogramming. We performed a single nucleofection with non-integrating oriP/EBNA1-based pluripotency plasmids in dissociated single-cell NPCs and plated on Matrigel substrate in reprogramming media containing FGF2 and LIF. The cortical NPCs early on (day 12) appeared to obtain morphology similar to that of pluripotent stem cell colonies, but after few days formed transient intermediates (**Fig. 2B**) that ultimately acquired differentiated and flat cellular morphologies. The pig spinal cord NPCs initially appeared to be malleable to plasmid-based reprogramming, as evidenced by typical iPSC morphology only 16 days post-nucleofection (**Fig. 2D**). However, after day 20 these promising colonies "slipped" into differentiated state in FGF2 and LIF containing iPSC media. **Again, we were unable maintain them in a pluripotent state. Therefore, we concluded that stronger and sustained expression of pluripotency reprogramming factors is required in Yucatan minipig cells.**

Recently, Sendai virus (SeV)-based reprogramming technology has been reported as an integration-free and highly efficient technique and is also sold as a kit (CytoTune kit, Life Technologies). SeV is a respiratory virus of mouse and rat and was first isolated in Japan in the early 1950s. SeV is an enveloped virus whose genome is a single chain RNA in the minus sense. Being an RNA virus, SeV does not integrate into the genome. We attempted to reprogram minipig spinal cord NPC using the SeV system containing vectors for OCT3/4, SOX2, KLF4, and C-MYC. Cells were dissociated and plated on MEF feeder layers 5 days after SeV viral transduction of spinal cord NPCs (**Fig. 3B**). Twelve days after viral transaction colonies resembling iPSC morphology appeared and were isolated for clonal expansion (**Fig. 3C and D**). We were successfully able to obtain 3 clonal SeV-piPSC lines (**Fig. 3E**). Colonies from a pig SeV-piPSC line is shown here with typical embryonic-stem cell like morphology with well defined borders and high nuclear-to-cytoplasmic ratio (**Fig. 3E'**). The SeV-piPSCs clones have been validated for pluripotency markers, including OCT3/4 (**Fig. 3F' and G'**). However, only a small number of cells were positive for the human iPS cell surface pluripotency markers, TRA-1-81, TRA-1-60, and SSEA-4. **These results are in concordance with recently published reports describing generation of pig iPS cells and production of chimeric offspring from them[3, 4].**

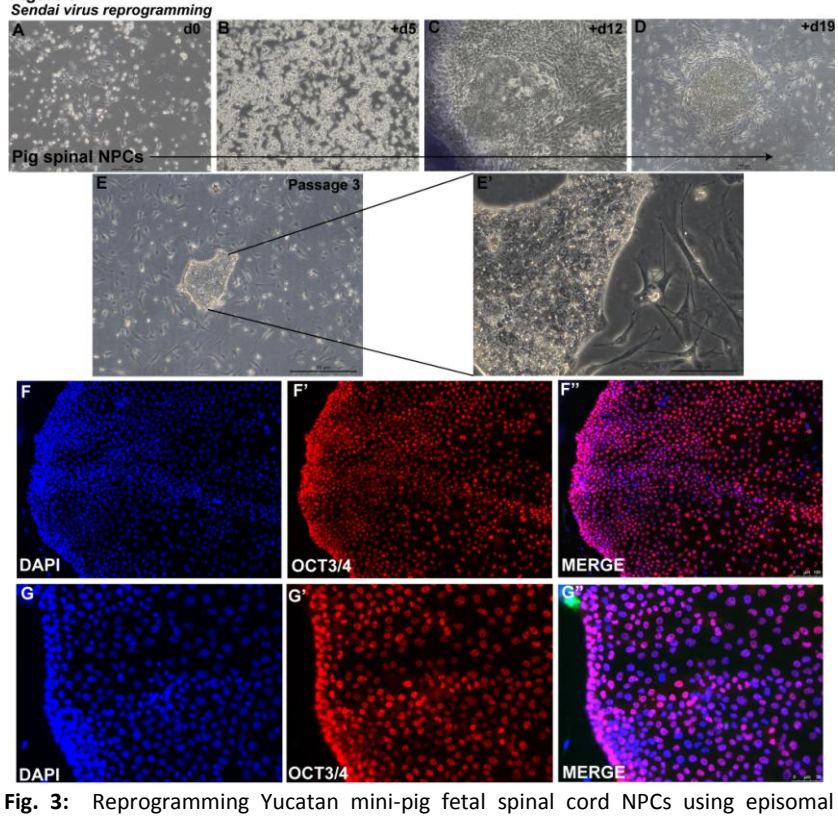

Fig. 3: Reprogramming Yucatan mini-pig fetal spinal cord NPCs using episomal Sendai virus approach. Phase contrast images showing morphology of (A-D) spinal cord NPCs plated on MEF feeders after transduction with SeV-pluripotency vectors. And colonies begin to emerge 12 days after transduction. (E and E') A representative iPSC colony isolated and expanded and showing (F-F'' and G-G'') nuclear co-localization of OCT3/4.

We are now banking and characterizing our SeV-piPSC lines for pluripotency and differentiation potential using the standard assays. We will also perform PCR analysis to confirm that there have been no integration events during the reprogramming. Following further expansion, minipig iPSC lines will be differentiated into neural progenitors akin to human NPCs. After gently lifting piPSC colonies and culturing in suspension medium containing high concentrations of EGF and FGF2, neural stem cell aggregates will be formed. These aggregates allow simple and rapid proliferation of multipotent NSC populations from iPSC colonies in the absence of feeder layers, expensive substrates or complex serum components. We will test these cells for relevant regional-specific markers prior to tissue transplantation. Using these SeV-pig iPSC-derived neural aggregate cells, we will establish and validate the autologous engraftment of minipig iPSC-derived astrocytes.

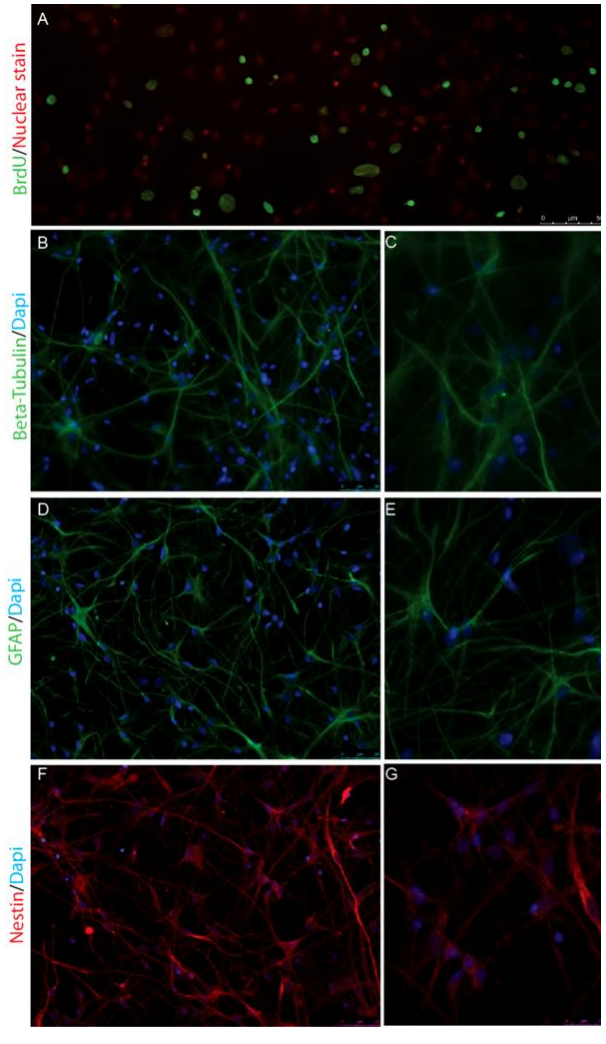

Fig. 4: Generation of E25 porcine cortical-derived progenitors. (A) Pig NPCs can be labeled with the cell proliferation marker bromodeoxyuridine (brdU; green) *in vitro* following 60 days in culture. Cell nuclei are counterstained with ethidium bromide (red) (B-G) Cortical progenitors express the immature neuronal marker β-tubulin (green) (B, C) as well as the intermediate filaments glial fibrillary acidic protein (GFAP;green) and Nestin (red) typically expressed by astrocytes (D, E) and neural progenitors (F, G) respectively. A, B, D, F: 20x magnification; C, E, G: 40x magnification. Cell nuclei in panels B-G are counterstained with Dapi (blue).

<u>GENERATION OF FETAL PIG-DERIVED NEURAL PROGENITORS</u>: As described above, we have encountered some hurdles in the generation of porcine iPS-derived NPCs. In order to pursue objective 2A of the grant proposal we have temporally circumvented this issue by isolating and expanding cortical and spinal neural progenitors from embryonic minipigs. In our first attempt, a pregnant sow was aborted at day 50 of gestation. Following the still birth of the embryos, cortices and spinal cord were isolated, mechanically dissociated and cultured to propagate neural progenitors using standard techniques[5]. Unfortunately, these neural progenitors did not expand as expected and cells senesced at low passage. We concluded that the lack of expansion and the early senescence of these pig neural progenitors were partially due to their "old" age at isolation but also to the long length of time required for the collection of the embryos following the abortion method. In our second attempt, following euthanasia of a pregnant sow, a C-section was performed and embryonic day 25 pig fetuses where rapidly isolated. Shortly thereafter, cortices and spinal cord were transferred to cell culture. Cortical-derived pig neural progenitors have been grown as spheres in media supplemented with epidermal growth factor (EGF) and fibroblast growth factor-1 (FGF-1), expanded by mechanical chopping and frozen down for future experiment at various passages. **Following 7 passages (60 days in culture) the cells continue to proliferate and**

express the appropriate markers typical of a neural phenotype following dissociation and 24 hours of differentiation without growth factors (Fig. 4). The survival of these cortical-derived pig neural progenitors following transplantation into the spinal cord of the minipig will be compared to that of human NPCs while waiting for the generation of minipig iPSC-derived astrocytes.

KEY RESEARCH ACCOMPLISHMENTS:

(Boulis Laboratory)

- **Equipment Purchase** (Sterrad surgical sterilization device and Stereology Software)

- **Aim 1** (all surgical procedures and behavioral analysis, as well as initial histological analysis have been completed)

DOD - Aim 1 (45 pigs)						
Status						
Number Pigs	Injection Site	Side	Cannula Type	Number Inj.	Volume Inj.	Dates
7	Cervical	Bilateral	Floating	20	10	Oct 11 (n = 4); Jun 12 (n = 3) same
5				30		Jan 12 (n = 1); Feb 12 (n = 3); May 12 (n = 1)
5				40		Feb 12 (n = 1); Apr 12 (n = 3); May 12 (n = 1)
7	Cervical	Bilateral	Floating	20	10	Oct 11 (n = 4); Jun 12 (n = 3) same
5					25	Oct 11 (n = 1); Dec 11 (n = 4)
5					50	Dec 11 (n = 1); Jan 12 (n = 3); May 12 (n = 1)
2					75	Aug 12 (n = 2)
3	Cervical	Bilateral	Hand-held	20	10	Jun 12
3					25	Jul 12
3					50	Jul 12 (n = 1); Aug 12 (n = 2)

(Svendsen Lab)

- Provided **cells for all procedures in Aim 1**, Year 1

- **Production of pig iPS cells**

- **Generation of fetal pig-derived neural progenitors**

REPORTABLE OUTCOMES:

1. Federici T, Hurtig CV, Burks KL, Riley JP, Krishna V, Miller BA, Sribnick EA, Miller JH, Grin N, Lamanna JJ, Boulis NM. (2012) Surgical Technique for Spinal Cord Delivery of Therapies. Demonstration of Procedure in Gottingen Minipigs. JoVE. e4371.

2. Quach DM, Boulis NM, Federici T, Hefferan MP, Wu S, Lee D, Lam T, Hayama KL, Hazel T, Marsala M, Wu MC, Johe K. Immune Tolerance after Cervical Transplantation of a Human Neural Stem Cell Line (NSI-566RSC) in Naïve Minipigs. Society for Neuroscience Abstract 555.27, 2012.

3. Miller JH, Hurtig CV, Grin N, Lamanna JJ, Federici T, Boulis NM. Porcine behavior and functional status following large volume cervical spinal cord injections. Abstract for AANS/CNS Spine Section meeting: Phoenix, AZ March 6-9, 2013.

CONCLUSION:

- In all experiments performed in Aim 1, all animals recovered to baseline behavior by post-operative day 14 despite the increasing number of injections and volume.

- The severity of acute transient morbidity should not be neglected, as well as the observation that the cord acutely swells with volume escalation making dural closure virtually impossible.

- The higher the number of injections the more likely is that the vasculature will interfere with a symmetric / linear pattern of injections. Staggered injections and reduced interval spacement of injections become a necessity.

- Hand-held injections can be performed in an incredibly quicker manner in comparison with injections using the platform. Nonetheless, accuracy and reflux are the main disadvantages of such technique.

- Histological analysis will further assess morbidity by determining whether there is a reduction in the number of motor neurons or not in grafted areas in comparison with intact segments.

- Histological analysis will also determine whether reflux occurs with volume escalation as well as with fast (hand-held) injections.

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APPENDICES:

Tarlov Score - Gait and motor function have been assessed according to a Tarlov's score. This scale provides objective criteria by which to evaluate ability to ambulate as a surrogate measure of motor function. The score is as follows:
(0) no voluntary limb function;
(1) only perceptible joint movement;
(2) active movement but unable to stand;
(3) to be able to stand but unable to walk;
(4) complete normal hind-limb motor function.

Video article
Federici T, Hurtig CV, Burks KL, Riley JP, Krishna V, Miller BA, Sribnick EA, Miller JH, Grin N, Lamanna JJ, Boulis NM. (2012) Surgical Technique for Spinal Cord Delivery of Therapies. Demonstration of Procedure in Gottingen Minipigs. JoVE. e4371.

Video Article

Surgical Technique for Spinal Cord Delivery of Therapies: Demonstration of Procedure in Gottingen Minipigs

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Abstract

This is a compact visual description of a combination of surgical technique and device for the delivery of (gene and cell) therapies into the spinal cord. While the technique is demonstrated in the animal, the procedure is FDA-approved and currently being used for stem cell transplantation into the spinal cords of patients with ALS. While the FDA has recognized proof-of-principle data on therapeutic efficacy in highly characterized rodent models, the use of large animals is considered critical for validating the combination of a surgical procedure, a device, and the safety of a final therapy for human use. The size, anatomy, and general vulnerability of the spine and spinal cord of the swine are recognized to better model the human. Moreover, the surgical process of exposing and manipulating the spinal cord as well as closing the wound in the pig is virtually indistinguishable from the human. We believe that the healthy pig model represents a critical first step in the study of procedural safety.

Video Link

The video component of this article can be found at http://www.jove.com/video/4371/

Protocol

1. Animal Use

Procedures demonstrated herein have been approved by the Emory University Institutional Animal Care and Use Committee (IACUC). Female Gottingen minipigs weighing approximately 15-20 kg are used.

2. Anesthesia

Animals are fasted approximately 12 hours prior to surgery. Animal sedation and anesthesia induction consist of a cocktail of intramuscular Ketamine (35 mg/kg), Acepromazine (1.1 mg/kg), and Atropine (0.04 mg/kg). Animals are then intubated and maintained on oxygen and 1-3% isoflurane general anesthesia. At this point, the back and head of each animal is shaved. Depth of anesthesia is monitored by the veterinary staff. Absence of interdigital, corneal and palpebral reflexes, as well as heart and respiratory rate, pulse oximetry, direct/indirect blood pressure, end-tidal carbon-dioxide measurements, and muscle tone/response to noxious stimuli are used to monitor depth of anesthesia.

3. Positioning

Animals are taken to the operating room and placed into a prone position on a frame custom designed to mimic patients positioning on a Jackson spinal surgical table. The frame utilizes adjustable slings that are placed under the chest and pelvis of the animal, allowing the abdomen to hang free and, therefore, minimizing pressure on the abdomen and chest and consequent epidural venous bleeding (**Figure 1**). The frame also provides external immobilization of the spine for the procedure [1].

Additionally, animals are placed on a heated re-circulating pad to maintain body temperature and a marginal ear vein catheterized for fluid administration and any necessary drug delivery during surgery. Finally, the surgical field is prepped with alcohol and Chlorhexadine or Betadine solution and surgical drapes are placed on the surgical field.

4. Laminectomy

An approximately 10-15 cm skin incision is performed and the paraspinal musculature is dissected off the spine bilaterally. Next, a dorsal multi-level laminectomy is performed. The lamina and spinous processes of three vertebrae overlying C3-C5 or L2-L4 segments are removed using rongeurs and a surgical drill.

5. Placement of the Spinal Derrick

We call Spinal Derrick the device designed for the delivery of (gene and cell) therapies into the spinal cord [2-5]. Detailed discussion on the design and evolution of this device can be found on Riley *et al*., 2011. [4]

To secure the device to the patient, percutaneous posts are placed through 1cm skin incisions above and below the primary incision and mounted to the lamina above and below the primary incision.

Next, two integrated retractors are attached to the four percutaneous posts above and below the incision site to expose the area of the spine that has undergone laminectomy.

6. Dural Opening

With the aid of a Woodson dental tool and an 11 blade, a 2.5 cm incision is made through the dura, exposing the spinal cord. The dura is reflected away from the pial layer using 4-0 Nurolon suture and secured to the deep paraspinal musculature.

Surgical patties are placed in the rostral and caudal extremes of the opening. These provide a partial barrier to cerebrospinal fluid flow and also provide a safe target for the surgeons to place suckers without damaging the cord. In humans, under surgical microscope magnification, the pial surface is dissected at this point. Due to technical limitations, this procedure is not required or feasible in animals.

7. Spinal Cord Injections and Lateral Displacement of the Spinal Derrick for Additional Injections

Immediately prior to injections, a bolus of Methylprednisolone (125 mg, IV) is given to prevent spinal cord swelling.

At this point, the platform rail system is attached and the side rails are adjusted to fit the appropriate length. The gondola is top-loaded onto the 2 bars and the Z drive is mounted on the universal joint. Next, the loaded cannula is placed onto the microdrive. Using the universal joint on the microinjection platform, the coronal and sagittal angles are adjusted to ensure a trajectory orthogonal to the surface of the spinal Injections follow the placement of the cannula. The needle is positioned accordingly medial to the dorsal root entry zone (DREZ). The DREZ is identified under 3.5X surgical loupe magnification and penetrated on an orthogonal trajectory to the cord surface at a point <1 mm medially.

In humans, a pre-operative MRI provides a baseline assessment of spinal cord dimensions for operative planning. Moreover, the thickness of the spinal cord is measured to determine the target depth of the ventral horn.

The suspension is infused at a depth of 4 mm from pial contact. A flange made of ultem plastic serves as a stopper on the pia surface to prevent the needle from advancing deeper than desired. Once the needle tip is positioned at the target, the rigid metal outer sleeve is pulled up, leaving the flexible tubing exposed. Once the injection is completed, the needle is left in place for an additional 1 min to prevent cell reflux up the cannula injection tract.

Care is taken to avoid surface vasculature by slightly adjusting the microdrive either laterality or rostro-caudally. Some bleeding from penetration sites may occur. When such bleeding is encountered, micro patties are placed over the bleeding puncture site and suction is applied to them to wick blood out of the cannula penetration site and prevent buildup in the cord. This reliably allows for the blood to coagulate. Cautery is avoided as is direct pressure.

Following needle removal, the stereotaxic apparatus is relocated to the next target site along the rostro-caudal axis, separated by 2 or 4 mm or as necessary to avoid visible blood vessels on the dorsal surface of spinal cord. This process is repeated as many times as proposed in a given study.

8. Floating Cannula

A custom infusion cannula of narrow diameter is used for the injections. The cannula consists of a 30-gauge beveled needle of fixed length connected to a 30-gauge flexible silastic tubing of variable length. The distal end is fitted with a Hamilton luer lock that is attached to a microinjector pump. The proximal silastic tubing is ensheathed within a 24-gauge rigid outer cannula that seats on the proximal end of the injection needle flange. This flange both seats the outer cannula and serves as a depth stop for the injection needle. For each injection, the appropriate volume of a therapeutic suspension is infused by a using a pre-calibrated MINJ-PD microINJECTOR pump (Tritech Research, Inc., Los Angeles, CA) at a rate of 5 µl per minute.

9. Closure

Once all injections have been made, the spinal derrick is gently removed and the incisions are closed in four layers. The dura is closed using a 4.0 Nurolon stitch, in a watertight fashion. 0 Vicryl suture is used for the deep muscular layer. Fascia is then closed with 0 Vicryl suture also in a watertight fashion. The dermal layer is finally closed with 2.0 Vicryl, with a running stitch. Skin closure is completed using a 3-0 Nylon suture.

10. Recovery and Pain Management

Animals are extubated and monitored for two hours following anesthesia recovery. Next, animals are transferred to individual cages and monitored at least once daily for food consumption, defecation, and micturition.

For pain management, a transdermal Fentanyl patch (75 mcg) is stapled on the back of the animals for three days of post-operative analgesia. Additionally, Buprenorphine (0.05 mg/kg, BID, IM) can also be given for up to three days post-operatively.

11. Results and Representative Outcomes

Clinical and behavior observations are performed before surgery and then recorded on Days 1 through 7 and weekly until endpoint according to the study design. Behavioral data is collected to assess neurological morbidity as previously described [6]. Sensory function is assessed by presence or absence of a withdrawal response to mechanical stimulus to the toes of front and hind limbs. Motor function follows the Tarlov score (**Table 1**): 0 - Paralysis, no movement; 1- Perceptible tonus in the hind limbs, slight movement; 2 - Movement in the hind limbs, but unable to sit or stand; 3 - Ability to stand and walk but ataxic and for short periods; 4 - Complete recovery, normal motor function.

Safety of the procedure is determined by the ability of an animal to return to pre-operative baseline. Transient neurological deficits should mostly resolve between post-operative days 1 and 7, with some variations depending on animals' breeds and procedure (number of injections, among other parameters). Permanent morbidity is defined by lasting neurological deficits which do not resolve by the time animals reach IACUC default endpoint (**Figure 2**).

Representative Results

Tarlov Score	
0	Paralysis, no movement
1	Perceptible tonus in the hind limbs, slight movement
2	Movement in the hind limbs, but unable to sit or stand
3	Ability to stand and walk but ataxic and for short periods
4	Complete recovery, normal motor function

Table 1. ***Tarlov Score.*** Neurological morbidity and recovery is assessed by scoring the animal's motor function.

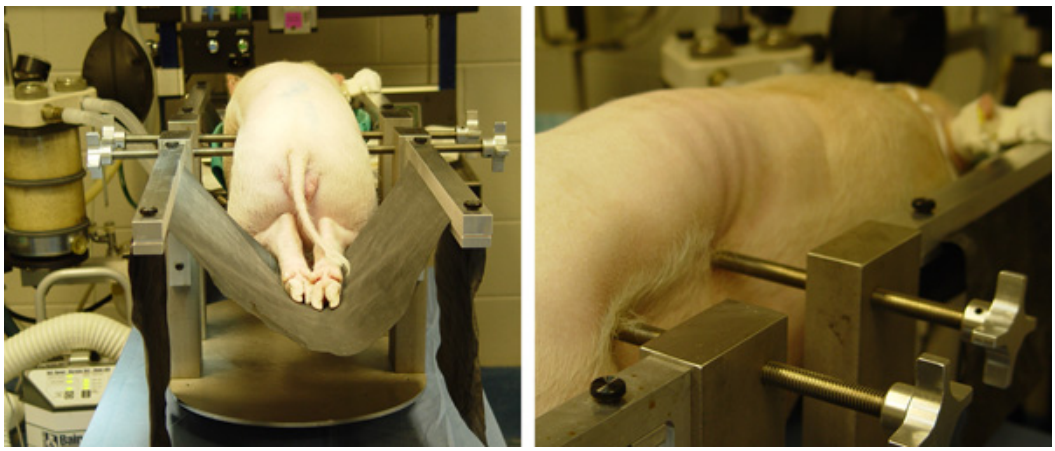

Figure 1. ***Table Positioning for Procedure.*** Animals are placed into a prone position on a frame custom designed to mimic patients positioning on a Jackson spinal surgical table. The frame utilizes adjustable slings that are placed under the chest and pelvis of the animal, allowing the abdomen to hang free and, therefore, minimizing pressure on the abdomen and chest and consequent epidural venous bleeding. The frame also provides external immobilization of the spine for the procedure.

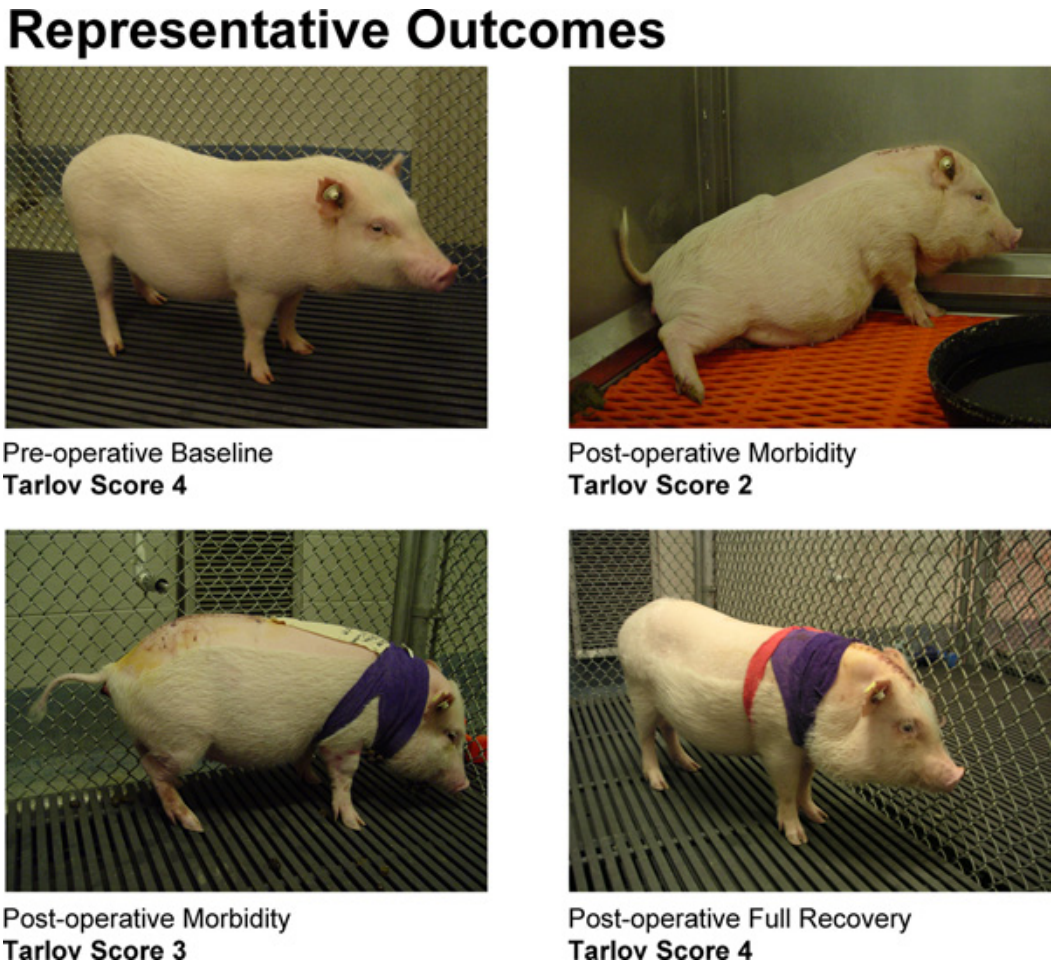

Figure 2. ***Motor Function Assessment and Representative Outcomes.*** Animals undergo a general neurological examination before surgery and on a regular basis following complete recovery from the procedure. Gait and motor function are assessed according to the Tarlov score. This scale provides objective criteria evaluating the animals' ability to ambulate as a surrogate measure of motor function. Safety of the procedure is determined by the ability of an animal to return to pre-operative baseline. Transient neurological deficits should mostly resolve between post-operative days 1 and 7, with some variations depending on animals' breeds and procedure (number of injections, among other parameters). Permanent morbidity is defined by lasting neurological deficits which do not resolve by the time animals reach IACUC default endpoint.

Supplemental

In case of cell therapies, prior to laminectomy and under anesthesia, a jugular vein 10F chronic catheter (Access Technologies, CCPS072106A) is placed for intravenous administration of immunosuppressants for the duration of the study. The neck of the animal is prepped and draped. The internal jugular vein is exposed surgically and cannulated with the catheter, which is secured with a 3-0 silk tie. The proximal end of the internal jugular is then ligated with a 3-0 silk tie. Next, the catheter is tunneled out of the neck skin dorsally and secured with 3-0 nylon stitches. Finally, the wound is irrigated and closed with a running 3-0 nylon stitch. Such procedure is not required in humans.

Discussion

Despite approval to proceed with the described technique in humans [7-9], critical questions remain to be answered in order for spinal cord therapies to succeed. A rigorous understanding of *spinal cord tolerance* to intraparenchymal injection is required to enable the planning and execution of trials developing therapies for demyelinating, degenerative, and traumatic spinal cord disease. Currently, there is no clear understanding of the number of injections that the large mammalian spinal cord can tolerate without transient and permanent morbidity. Similarly, spacing of injections is likely to affect morbidity. Moreover, macroscopic (*e.g.*, ventilation-related or inadvertent patient movement) and microscopic (*i.e.*, oscillation with both ventilation and cardiac pulse) spinal cord movements pose risks to the spinal cord during injection. An understanding of the threshold for morbidity in a large animal model will aid dosing calculations for all spinal cord therapy programs. Our translational spinal cord transplantation laboratory is available to help preclinical development programs of all teams currently designing trials for spinal cord application.

Disclosures

Dr. Boulis is the inventor of devices to enable safe and accurate injection of the human spinal cord. Neuralstem, Inc. has purchased an exclusive license to this technology. Dr. Boulis received an invento's share of this fee, and has the rights to royalty payments for distribution of this technology. Other authors have nothing to disclose.

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